Environment Canada Environnement

NWRI CONTRIBUTION

Vol: 89 No: 156 Date: 890800

2038886K

ODFF EXC 1

RRB-89-61

MUTATOX TEST: A NEW ENVIRONMENTAL IMPACT ASSESSMENT PROCEDURE FOR WATER AND SEDIMENT

by
K.K. Kwan, B.J. Dutka
S.S. Rao and D. Liu

Rivers Research Branch
National Water Research Institute
Canada Centre for Inland Waters
Burlington, Ontario, L7R 4A6

August 1989
NWRI Contribution #89-156

ABSTRACT

In this study, Yamaska River water and Milli-Q water and organically extracted sediment extracts were used to evaluate the sensitivity of a new genotoxicity screening test, the Mutatox test. Also in this study, the samples were tested for acute and chronic toxicity using the following screening test procedures: Microtox, Daphnia magna, Cereodaphnia reticulata and ATP-TOX System.

The Mutatox test is based on the use of a dark mutant strain of Photobacterium phosphoreum and is sensitive to chemicals which are (1) DNA damaging agents, (2) DNA intercalating agents, (3) DNA synthesis inhibitors and (4) direct mutagens.

In this study, the Mutatox test was found to be a simple-to-perform sensitive procedure which added greater scope to the battery of tests approach. Preliminary indications are that this procedure may prove to be on the more responsive and valuable tests in the "battery of tests" approach to environmental screening.

RÉSUMÉ

Dans la présente étude, des échantillons d'eau de la rivière Yamaska, d'eau Milli-Q et de sédiments organiques ont été utilisés pour déterminer la sensibilité d'un nouvel essai de dépistage de la génotoxicité, l'essai Mutatox. De plus, les échantillons ont été analysés en vue d'évaluer la toxicité aiguë et chronique par les méthodes de dépistage suivantes: Microtox, Daphnia magna, Cereodaphnia reticulata et le système ATP-TOX.

L'essai Mutatox consiste en l'utilisation d'une souche mutante de Photobacterium phosphoreum ne produisant pas de lumière et sensible aux produits chimiques qui sont des 1) agents endommageant l'ADN, 2) des agents s'intercalant dans l'ADN, 3) des inhibiteurs de synthèse de l'ADN et 4) des agents mutagènes proprement dits.

L'essai Mutatox s'est révélé une méthode simple et sensible qui a élargi l'approche axée sur la série d'essais. Les résultats préliminaires indiquent qu'il pourrait s'agir de l'essai le plus sensible et le plus utile de la série d'essais effectués pour surveiller l'environnement.

MANAGEMENT PERSPECTIVE

Industrial pollutants and toxicants such as herbicide, insecticide, fertilizers and car exhaust fumes affect the aquatic biota in many ways and at different levels. It is conceived that the "battery of tests" approach using several different short-term biological tests would be preferred in monitoring our environment.

In this study, the Yamaska River water and Milli-Q water and organic sediment extracts were used to evaluate the sensitivity of a newly developed genotixicity screening test, the MUTATOX test. The Mutatox test was found to be a simple, sensitive and reliable procedure and warrants inclusion into the "battery of tests" approach for environmental screening.

PERSPECTIVES GESTION

Les polluants industriels et les substances toxiques comme les herbicides, les insecticides, les engrais et les gaz d'échappement des automobiles influent sur le biote aquatique de plusieurs façons et à des degrés divers. Il est concevable que la série d'essais axée sur l'utilisation de plusieurs essais biologiques à court terme soit la plus appropriée pour surveiller notre environnement.

Dans la présente étude, des échantillons d'eau de la rivière Yamaska, d'eau Milli-Q et de sédiments organiques ont été utilisés pour évaluer la sensibilité d'un nouvel essai de dépistage de la génétoxicité, l'essai MUTATOX. Cette méthode est simple, sensible et fiable et mérite d'être incluse dans la série d'essais pour la surveillance de l'environnement.

INTRODUCTION

In response to the ever increasing stresses to our environment and the realization that there is no single criterion by which potential of hazard (either to the environment or man) of any contaminant can be assessed, a multitude of biological assay procedures have been developed or are being developed to assess toxicant impacts in aquatic systems.

As industrial pollutants and toxicants such as oil refinery wastes, herbicides, insecticides, fertilizers, and car exhaust fumes affect aquatic biota systems at different levels and in many ways, it is acknowledged that the "battery of tests" approach utilizing several different short-term biological tests would be preferred in any monitoring scheme.

In our studies of rivers and lakes and their sediments, the "battery of tests" approach has been used to assess the degree and extent of contaminant/toxicant effects (Dutka, 1988; Dutka et al., 1988; Dutka and Kwan, 1988). As new promising toxicant screening procedures become available, they are evaluated for potential inclusion in our battery of tests. Recently, the Microbics Corporation proposed a new biological test which measures the ability of a chemical or sample to induce a genetic or epigenetic change in the test organism Phosphobacterium phosphoreum. This procedure, called the Mutatox test, was evaluated, using Yamaska River Basin waters and sediments, as a potential candidate for our battery of tests approach

to screen for environmental toxicants. The results of this investigation are presented.

METHODS

Site

Nine sites in the Yamaska River Basin, Quebec, Canada, representative of the industrial, urban and rural pollution sources in the basin, were selected for this study. Site locations, descriptions and sediment descriptions have been described in an earlier paper (Dutka et al., 1989).

Sample Collection

Sediments at each site were collected in triplicate (15 metres apart) using a shovel or Ekman dredge. Frequently, it was necessary to shovel or ekman many times before sufficient surface sediment (1 to 2 cm layer) was collected. At each sampling site the sediments were well mixed, placed into appropriate containers and refrigerated. One litre surface water samples were collected at each site (3 per site) and preserved at 4°C for concentration procedures and toxicant screening tests. Water samples for toxicant screening tests (with the exception of Daphnia and Cereodaphnia tests) were tested after being concentrated 10X by flash evaporation at 45°C.

Sediment Extraction

Five hundred grams wet weight of sediment was extracted (1:1) with Milli-Q water (4 cartridge system, 1 Super C carbon cartridge, 2 Ion ExTM cartridges, 1 Organex-Q cartridge and a Milli-StakTM filter with a glass distilled water feed) by mixing sediment and water and shaking vigorously for three minutes. The mixture was centrifuged at 10,000 rpm in a refrigerated centrifuge for 20 minutes. The supernatant was used in toxicity screening tests. Then the water extracted sediment in 100 gram portions was freeze-dried, then weighed on prefired foil (550°C overnight). Following the procedure outlined by Dutka and Kwan (1988), the sediment was extracted with DCM and concentrated into 1 mL 100% DMSO at ratio of 100 g wet weight sediment per 1 mL 100% DMSO.

Screening Tests

The Microtox test was performed using the luminescent bacterium Photobacterium phosphoreum and the procedure detailed in the Microtox Operation Manual (1982) with a 15 min contact time (Dutka and Kwan, 1984). ATP-TOX System a toxicity screening test based on the inhibition of bacterial growth and luciferase activity, was applied to water and sediment extracts (Xu and Dutka, 1987).

A 48 hr. Daphnia magna test, using 10 organisms per sample and sample dilution, was used to test all water and sediment extract samples (APHA, 1985). The seven day Ceriodaphnia reticulata 3-brood life cycle chronic toxicity test using four cladocerns per sample or dilution was also used in this study (Rao, 1988).

A new test, the Mutatox test, based on the use of a dark mutant strain of Photobacterium phosphoreum M169, to screen for the presence of genotoxic agents was evaluated in this study. This test will pick up chemicals which are (a) DNA damaging agents, (b) DNA intercalating agents, (c) direct mutagens which either cause base substitution or are frame shift agents, and (d) DNA synthesis inhibitors (Microbics, 1988). These chemicals will restore the light emitting stage of the strain which can be measured in a modified Beckman Microtox Model 2055 analyzer. The test procedures are similar to those followed in the Microtox test with incubation of M169 cells, cell media and sample being carried out at 20-24°C for 18 hr. Light level is read after 18 $\pm$ 1 hr contact and compared to negative controls (solvent and sodium azide). Details of the procedure are as follows: (1) prepare 14 12 x 50mm test cuvettes, (2) add 1.0 mL assay mixture into cuvette #1 (assay mixture = media + culture in 100:1 ratio), (3) add .5 mL assay mixture into cuvettes 2-14, (4) add .02mL sample into cuvette #1, (5) dilute sample in cuvette #1 into cuvettes 2-10 (1:2 serial dilutions) (6) incubate cuvettes at 20 - 24°C and (7) read light level for each cuvette at 18$\pm$ hr. A positive control, 4-nitroquinoline-N-oxide, was also used to establish the stability of the testing

procedure. Although the test can also be performed with S-9 addition, in this study S-9 was not used. All toxicant or genotoxic data are the mean of duplicate samples.

Ranking Scheme

Previously, a ranking scheme was developed to award points for specific data values (Dutka, 1988) in order to rank samples (water and sediment extracts) from those of most concern to those of least. In this study, an edited version of the ranking scheme (Table 1) was used to simplify data presentation. For the Mutatox test, a point allocation scheme based on observed results was not able to be established since we, as yet, do not have a sufficient data base to establish a point value for specific environmental sample values. Therefore in this presentation, the Mutatox data are presented as the number of times the number of revertants are greater than the controls. In this study, only those samples having a genotoxic effect 3 times (3X) the control number of revertants, are considered positive and are discussed.

RESULTS AND DISCUSSION

In environmental samples, specifically water and sediment, it is extremely rare to encounter a toxicant or genotoxicant in pure form. Invariably a toxic or genotoxic effect is due to the combined effect of many chemicals in a variety of combinations and concentrations,

which trigger the effects noted in toxicant screening tests. Usually, these effects are additive and perhaps multiplied (Dutka and Kwan, 1984). In the Yamaska River, it is known (Tate, 1972) that these waters have been/are polluted by a great variety of industrial chemicals, pesticides, herbicides, farm land runoff, and urban sewage treatment plant discharges.

These waters and sediments were expected to provide a great variety of triggering agents for the "battery of tests" approach. Some of the summarized results can be seen in Tables 2, 3 and 4. Table 2 summarizes the number of times each test indicated the presence of toxic or genotoxic substances in the three sample types. In viewing this table, it must be remembered that the Daphnia and Ceriodaphnia tests were performed on natural, unconcentrated water samples while the other tests were performed on concentrated water samples (10X). In Table 2 columns 1 and 2, it can be seen that the ATP-TOX System and Daphnia magna tests were the most responsive tests with the Daphnia test possibly being the most sensitive. The Mutatox test was found to be positive in 36 out of the 65 water samples tested, a strong indication that its' response is being triggered by chemicals different from those primarily affecting the other four tests. This supposition is supported by the data presented in Table 3. We believe these observations are very important in helping one select the make-up of a "battery of tests" to screen environmental samples. Any test which can be shown to be responding independently of the other tests, perhaps to different classes or mixtures of

chemicals, is an important test. It is in this way that fewer toxicant effects will be missed. The Mutatox test, in this study seems to not only provide greater scope to the battery of tests approach, but it also indicates the presence of genotoxic agents.

In the DCM-DMSO sediment extracts, the Microtox, Daphnia, Cereodaphnia and ATP-TOX System tests all indicated that each sample tested contained toxicants while the Mutatox test indicated the presence of genotoxic effects in one third of the samples. This observation may be indicative that (a) the majority of the genotoxic agents in the Yamaska River are water soluble, and that there are at least two classes of genotoxic agents in the Yamaska River basin samples or (b) since it is well known that certain compounds do not cause mutation unless subjected to metabolic enzyme actions normally occurring in the body, it is possible that if S-9 (induced rat liver homogenate) were used, more positive responses would be found.

Selected results illustrating the variability of the responses observed in this study are shown in Table 4. The data in this table again indicate the independent nature of the Mutatox test in relation to the other tests which screen for acute and chronic toxicity in water and sediment samples.

The specificity and potential of chemicals to cause spontaneous dark varients of luminous bacteria to revert to the luminous form, which was noticed by Nealson and Hastings (1979), eventually led to the development of the Mutatox test by the Microbics Corporation. In 1980, Ulitzur *et al.*, isolated a spontaneous dark varient of the

luminous bacterium Photobacterium leiognathi and found that it was possible to detect mutagenic compounds at concentrations 100 times lower than that detected by the Ames Test. These studies led to the development of an engineered strain of Photobacterium phosphoreum with a selective capability to screen for chemicals or mixtures of chemicals able to induce genetic or epigenetic changes in the testing organism (Microbics, 1989). This organism's sensitivity has been evaluated against a variety of known and suspected carcinogens as well as the Ames' Test. In Table 5, an example of the sensitivity of the Mutatox procedure to various chemicals is presented. From the data presented in this paper (Table 4) it can be seen that the Mutatox test is able to provide indications of the presence of potentially hazardous contaminants which several of the other "battery of tests" procedures were not able to.

The results of this study provide strong support for using the Mutatox test in any "battery of toxicant screening tests" to screen for acute and chronic toxicants and genotoxicants. We also believe that with the addition of S-9 (rat liver homogenate) to the testing protocol, this simple, easy to perform test will be an invaluable member of any battery of toxicant screening tests for environmental chemicals with mutagenic potential.

REFERENCES

American Public Health Association. 1985. Standard Methods for the Examination of Water and Wastewater. 16 ed. American Public Health Association, New York.

Beckman Instruments Inc. 1982. Microtox system operating manual. No. 015-555-879.

Dutka, B.J. and K.K. Kwan. 1984. Studies on a synthetic activated sludge toxicity screening procedure with comparison to three microbial toxicity tests. In: Toxicity Screening Procedures Using Bacterial Systems. D. Liu and B.J. Dutka, eds., Marcel Dekker Inc., New York, pp. 125-138.

Dutka, B.J. 1988. Priority setting of hazards in waters and sediments by proposed ranking scheme and battery of tests approach. Angewandte Zoologie, 20:303-316.

Dutka, B.J. and K.K. Kwan. 1988. Battery of screening tests approach applied to sediment extracts. Toxicity Assessment, 3:303-314.

Dutka, B.J., K. Jones, K.K. Kwan, H. Bailey, and R. McInnis. 1988. Use of microbial and toxicant screening tests for priority site selection of degraded areas in water bodies. Wat. Res. 22:503-510.

Dutka, B.J., K.K. Kwan and S.S. Rao. 1989. An ecotoxicological and microbiological study of the Yamaska River, NWRI Contribution #89-147, Burlington, Ontario, Canada.

Microbics Corporation. 1988. Personal communication. Microbics, Carlsbed, California.

Nealson, K.H. and J.W. Hastings. 1979. Bacterial bioluminescence: It's control and ecological significance. Microbiol. Rev., 43:496-518.

Rao, S.S. 1988. Ceriodaphnia reticulata seven-day survival and reproduction test. Toxicity Assessment, 3:239-244.

Tate, D.M. 1972. Economic and Financial Aspects of Wastewater Treatment in the Yamaska River Basin, Quebec. Social Science Series No. 3. Inland Waters Directorate, Water Planning and Management Branch, Ottawa, Canada.

Ulitzur, S., I. Weiser and S. Yannai. 1980. A new sensitive and simple bioluminescence test for mutagenic compounds. Mut. Res., 74:113-124.

Xu, H. and B.J. Dutka. 1987. ATP-TOX System. A rapid sensitive bacterial toxicity screening system based on the determination of ATP. Tox Assess., 2:149-166.

Table 1. Modified point awarding scheme for sample ranking, based on contained hazards.

ATP-TOX System % Inhibition per mL 10X Water Sample 1:1 Milli Q Water Sediment Extract 1% DMSO Sediment Extract	Microtox EC_{50} % per mL 10X Water Sample 1:1 Milli Q Water Sediment Extract 1% DMSO Sediment Extract	Points	*Daphnia magna* EC % per mL 1:1 Milli Q Water Sediment Extract 1% DMSO Sediment Extract	Points	*Daphnia magna* EC % per mL Water Sample	Points
1 - 30	40.0+	1	EC_{20} at 100%	1	EC_{20} at 100%	1
31 - 60	40.0 - 25.0	3	EC_{40} at 100%	2	EC_{40} at 100%	2
61 - 90	24.0 - 10.0	5	EC_{50} at 100%	4	EC_{50} at 100%	5
91 - 99	9.0 - 1.0	7	at 75%	5	at 75%	7
100	0.9	10	at 50%	6	at 50%	8
			at 25%	8	at 25%	10
			at 10%	10		

Ceriodaphnia reticulata % of water sample, 1:1 Milli Q water sediment extract 1% DMSO Sediment extract able to produce reproduction inhibition	Points
100%	2
50%	3
10%	5
1%	10
0.1%	15
0.01%	20

Table 2. Number of times each test was positive out of total samples tested.

Test	Type of Sample		
	Water	Milli-Q Water Extracted Sediment	DCM-DMSO Extract Sediment
Mutatox	36/65	5/46	15/45
Microtox	3/65	2/46	46/46
Daphnia magna	44/65	22/46	46/46
Ceriodaphnia reticulata	0/65	5/46	8/8*
ATP-TOX System	64/65	29/46	46/46
Number of samples all tests positive	3	0	14
Number of samples all tests negative	1	9	0

* - only 8 samples were tested with Cereodaphnia

Table 3. Illustrating the number of samples in which Mutatox and the other screening tests were both positive.

Test	Type of Sample		
	Water	Milli-Q Extracted Sediment	DCM-DMSO Sediment Extract
Microtox/Mutatox	$\frac{3}{36}$	$\frac{0}{5}$	$\frac{15}{45}$*
Mutatox/Daphnia	$\frac{32}{44}$	$\frac{2}{22}$	$\frac{15}{45}$
Ceriodaphnia/Mutatox	$\frac{0}{36}$	$\frac{1}{5}$	$\frac{1}{8}$**
Mutatox/ATP-TOX System	$\frac{36}{64}$	$\frac{3}{29}$	$\frac{13}{45}$

* - one sample was not tested by Mutatox procedure
** - only 8 samples were tested with Ceriodaphnia

Table 4. Selected sampling site data showing typical responses to battery of toxicant screening tests.

Sampling Site	Mutatox x Times Control Level of Revertants	Microtox Point Score[2]	Daphnia Point Score	Ceriodaphnia Point Score	ATP-TOX System Point Score	Type of Sample
11A	6X[1]	2.5[1]	3[1]	0[1]	5[1]	Surface Water
12C	42X	0	0.5	0	3	Surface Water
15A	5X	0.5	1	0	1	Surface Water
15B	4.5X	0	0.5	0	1	Surface Water
30A	0	0	0	0	3	Surface Water
31A	4.5X	0	0.5	0	5	Surface Water
10C	7X	0	0	0	0.5	Milli Q extracted sediment
11A	7X	0	0	0	0	Milli Q extracted sediment
11B	6X	0	2	0	0.5	Milli Q extracted sediment
11C	7X	0	0	0	0	Milli Q extracted sediment
15A	7X	0	1	10	0.5	Milli Q extracted sediment
31C	0	0	4	0	0	Milli Q extracted sediment
5A	0	7	4	5	2	DCM-DMSO extract
5B	12X	6	7	-[3]	2	DCM-DMSO extract
10A	13X	5	6.5	3	4	DCM-DMSO extract
11C	15X	6	7	-	3	DCM-DMSO extract
15B	0	10	7	-	1	DCM-DMSO extract
30A	3X	5	5.5	20	3	DCM-DMSO extract
31C	12X	5	7	-	2	DCM-DMSO extract

[1] = average of 2 tests
[2] = see Table 1
[3] = sample not tested

Table 5. Sensitivity of Mutatox Test and Ames' Test to selected chemicals[1].

Chemical	Test		
	Carcinogenicity	Ames	Mutatox
Dioxane	+	-	-
Ethylmethanesulfate	+	+	+
8-OH Quinoline	-	+	+
Methanol	-	-	-
Phenol	-	-	+
Reserpine	+	-	-
Safrole	+	-	+
Cyclophosphamide	+	+	-
9-aminoacridine	?	+	+
Acridine orange	?	+	+
Benzidine	+	+	+
Eugenol	+	-	+
1,2 Benzanthracene	+	+	+
Propyl gallate	?	-	+

[1]Source, Microbics, Carlsbad, California